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13. ABSTRACT (Maximum 200 Words)
AND-34 is a murine protein that binds by a cdc25-like GDP exchange factor domain to the focal adhesion docking protein p130Cas. Over-expression of either of the human homologues of AND-34 and p130Cas, BCAR3 and BCAR1, respectively, have been reported to induce resistance to antiestrogens in breast cancer cell lines. In our work over the last two years, we have ascertained that over-expression of AND-34 leads to activation of the Rho family GRPases Cdc42 and Rac. We further have found that transfection of a constitutively active form of Rac induces antiestrogen resistance in breast cancer cell lines. Further, we find that BCAR3-mediated cyclin D1 up-regulation in breast cancer cell lines is dependent on both Rac and a Rac effector protein, PAK1. This work, which is described in an accompanying Cancer Research article that is in press for Oct. 15, 2003, suggests that one mechanism by which breast carcinoma could develop antiestrogen resistance is by up-regulation of Rac and PAK1-dependent signaling pathways. In further work over the past year, we find that both the SH2 domain and the GDP exchange factor-like domain of BCAR3 are required for antiestrogen resistance, as transfection of mutants lacking these domains fails to allow growth of ZR-75-1 cells in the antiestrogen ICI 182,780. Finally, we have detected by Western analysis expression of BCAR3 in primary human breast carcinomas but not in normal mammoplasy specimens. Our efforts to detect BCAR3 by immunohistochemistry are ongoing.

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Introduction

This is the second annual report for this project.

In the first year of this grant support, we made several exciting observations that may help to explain how it is that over-expression of BCAR3 induces anti-estrogen resistance in estrogen dependent human breast cancer cell lines. Specifically, we demonstrated that over-expression of BCAR3 activated Rac and Cdc42, as well as the cyclin D1 promoter. We also showed that BCAR3's ability to activate the cyclin D1 promoter is Rac and PAK1-dependent. With regard to our stated goals in our Statement of work, we generated a system to study BCAR3-mediated antiestrogen resistance by reproducing Dorssers work in ZR-75-1 cells. Secondly, we generated a polyclonal antisera that could detect human BCAR3 and used it to demonstrate high levels of BCAR3 in a human breast carcinoma specimen.

In the second year of this grant support, we focussed on Task #1 and Task #3. For Task #1,we generated stable transfectants of full length, GEF-like domain deleted or SH2 domain deleted BCAR3 in the tamoxifen-sensitive breast cancer cell line ZR-75-1, followed by analysis of anti-estrogen resistance. As discussed below, we found that while optimal antiestrogen resistance was generated in cells transfected with full length BCAR3, significant resistance was not seen in either the GEF-like domain deleted transfectants or the SH2 domain-deleted transfectants, suggesting that the GEF-like domain and the SH2 domain are required for BCAR3-mediated antiestrogen resistance.

For task #3, given that the anti-BCAR3 antisera generated in year #1 was not optimal for immunohistochemical analysis, we made a new anti-BCAR3 antisera in rabbits. We have now tested this antisera both by Western analysis and by immunohistochemistry. We find that the new antisera sees BCAR3 well in p130Cas immunopreciptates and that it detects BCAR3 by immunohistochemistry in the breast cancer cell line 578-T. Our plan is to now test this antisera on formalin-fixed breast cancer and normal mammoplasty slides.

Body

Task 1. To determine whether AND-34/BCAR3's GEF activity is required for its ability to induce resistance to tamoxifen-induced growth arrest in breast cancer cell lines.

a. Develop a series of AND-34 expression construct plasmids in which residues in the GDP exchange factor domain expected to be critical for catalytic activity will be mutated to alanine using the Quick-Change mutagenesis kit (Months 1-7).

Progress: At this time, we are uncertain whether BCAR3 is itself a GDP exchange factor or an adapter protein. In experiments in another model system, lymphoid cells, we have seen activation of Cdc42 in the absence of a GEF domain,

suggesting that perhaps the GEF-like domain's primary function is activation of signaling as a result of binding to another protein, such as p130Cas. For this reason, as discussed below, we plan to focus on trying to identify the proteins to which BCAR3's SH2 domain binds.

b. Perform GDP exchange assays to identify AND-34 mutants which have lost the ability to catalyze Ral GDP exchange in Cos-7 cells by pulldown assay (Months 7-18).

Progress: As discussed above, we are not sure at this time that BCAR3 is itself a GDP exchange factor. Efforts to identify Ral or Rap activation in breast cancer cells after BCAR3 over-expression did not show such activation. Thus, as discussed below, we instead plan to focus on trying to identify the proteins to which AND-34/BCAR3's SH2 domain bind to in hope of better understanding how BCAR3 over-expression induces antiestrogen resistance.

c. Develop a system to demonstrate the ability of AND-34/BCAR3 to mediate tamoxifen resistance in ZR-75-1 cells (Months 1-12). Vector only or AND-34 transfected ZR-75-1 cells will be assessed for growth in the presence of 4-hydroxy-tamoxifen.

Progress: **This portion of work has been successfully completed**, as shown in manuscript that has been accepted in Cancer Research and is slated for publication on October 15th, 2003 (Appendix A). We first demonstrated that our ZR-75-1 cell breast cancer cell lines were sensitive to growth inhibition by the antiestrogen ICI 182,780 (data not shown but documented in year #1 annual report). In our hands, this pure antiestrogen gave clearcut growth inhibition in ZR-75-1 cells, while tamoxifen did not. ICI 182,780 is now in clinical use and is a clinically relevant test of the ability of breast cancer cells to grow in the absence of estrogen (tamoxifen is a mixed rather than pure estrogen antagonist). Our subsequent work will carried out with ICI 182,780. Of note, the human breast cancer cell line 578T was resistant to growth inhibition by ICI 182,780 (data not shown but documented in the year #1 annual report).

We subsequently obtained stable transfectants of ZR-75-1 cells expressing either HA-BCAR3 (Figure 4, Appendix A) or vector only. Three stable transfectants of each type were grown for 13 days in 100 nM ICI 182,780. As shown in Figure 6D, cells over-expressing BCAR3, but not cells stably transfected with vector only, were able to grow in the presence of the ICI 182,780.

d. Test the ability of AND-34 GDP exchange factor domain mutants to mediate tamoxifen resistance in ZR-75-1 cells (Months 12-36).

Progress: **This portion of work has been successfully completed**, as shown in the accompanying Figure 1. We obtained stable transfectants of ZR-75 cells over-expressing either control vector, full length BCAR3, BCAR3 lacking the

SH2 domain or BCAR3 lacking the GEF domain. Transfectants were then grown for two weeks in 100 nM ICI 182,780, a pure antiestrogen, followed by cell counting in triplicate. The data from the four sample groups were then assessed by ANOVA analysis, with subsequent use of the Bonferroni method for pairwise comparison using the SAS software statistics program. As shown in Figure 1, only transfection with full-length BCAR3 allowed growth in the presence of the ICI 182,780, while transfection with the two deletion mutants resulted in no significant difference in growth from that observed in cells transfected with the vector alone.

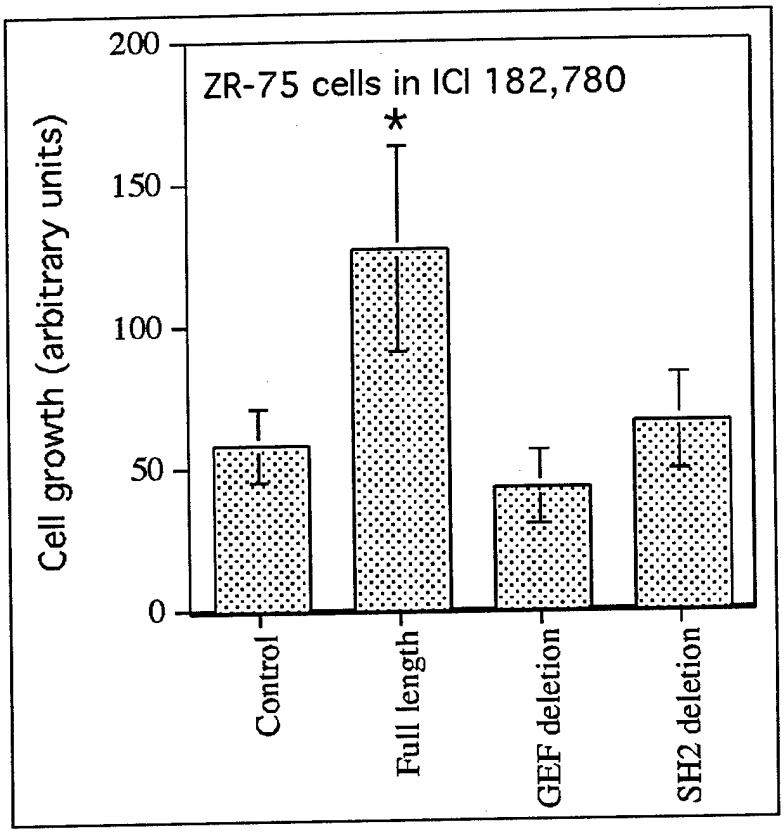

Figure 1: ZR-75-1 breast cancer cells were transfected with control vector, full length BCAR3, or constructs in which either the SH2 domain or the GEF-like domain of BCAR3 were deleted. Stable transfectants were obtained. The four classes of stable transfectants were then grown in 100 nM ICI 182,780, a pure antiestrogen, for two weeks. Clones were counted in triplicate. The total cell numbers, here shown in arbitrary units, were then compared using ANOVA analysis, with a secondary Bonferroni pairwise analysis. Only full-length BCAR3 transfectants were found to grow better than control cells in a statistically significant fashion.

Task 2. To determine whether AND-34 over-expression induces Src family kinase activation.

a. Develop a src kinase assay using immunoprecipitation, incubation with 32P and enolase, electrophoresis and PhosphoImager quantitation of enolase phosphorylation. Utilize constitutively active Src and/or EGF as positive controls (Months 1-6).

Progress: This work has not yet been performed. We will initiate this work within the coming year.

b. Test whether over-expression of wildtype AND-34 induces Src kinase activation in Cos-7 and HEK-293 cells (Months 7-12).

Progress: This work has not yet been performed. We will initiate this work within the coming year.

c. If AND-34 mediates Src activation, examine the ability of AND-34 SH2, Proline-domain, or GEF domain defective mutants to activate Src (Months 12-36).

Progress: This work has not yet been performed. We will initiate this work in the coming year.

d. If AND-34 does not mediate Src activation, determine whether other Src family members are activated by AND-34 over-expression (Months 12-36).

Progress: This work has not yet been performed. We will initiate this work in the coming year.

Task 3. To determine whether AND-34/BCAR3 is over-expressed in a subset of human breast carcinoma specimens.

a. Generate polyclonal rabbit antisera to: 1) AND-34/BCAR3 peptides contained within the human BCAR3 sequence; 2) A GST-AND-34 fusion polypeptide in which the GST tag has been removed by thrombin cleavage following purification of the chimeric protein (Months 1-8).

Progress: **This portion of work has been successfully completed**, as shown in part in Figure 4B of the accompanying Cancer Research article (Appendix A). We generated polyclonal rabbit antisera against BCAR3. ELISA analysis verified that the antiserum was of high titer. The antiserum was peptide immunopurified. The studies for specificity are described below. In contrast, although we made a GST-AND-34 fusion protein and utilized it for rabbit immunization, the resulting product did not give a useful reagent when assessed with the quality control measures discussed below.

b. Test the antisera for their utility in Western blot analysis using lysates from either wild-type MCF-7 cells (negative control) or AND-34 (positive control) (Months 9-12).

Progress: **This portion of work has been successfully completed**. MCF-7 cells transfected with BCAR3, then lysed and immunoprecipitated with anti-p130Cas, contained a 100 kD immunoreactive band which was not present in vector only-transfected MCF-7 cells (data not shown). Similarly, as shown in Figure 4B of Appendix #1, while 578T cells contained both BCAR3 transcript and BCAR3 protein, as judged by a 100 kD immunoreactive protein in p130Cas immunoprecipitates, ZR-75-1 cells contained neither BCAR3 transcript not BCAR3 protein.

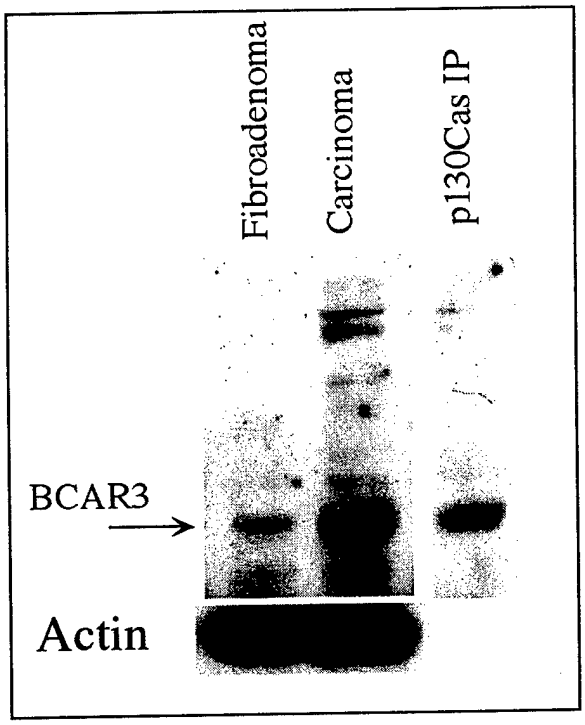

Figure 2: Tissue from a reduction mammoplasty, a breast fibroadenoma, and a breast carcinoma were immunoblotted for BCAR3. In addition, the carcinoma sample was immunoprecipitated with anti-p130Cas prior to immunoblotting. As a control for loading of cytoskeletal proteins, the same blot was then analyzed for actin.

c. Test the antisera for their utility in immunohistochemical analysis after growing and fixing wild-type or AND-34 transfected MCF-7 cells on tissue culture slides (Months 9-15).

Progress: This year, we carried out immunohistochemical analysis of 578T cells, a cell line that we have established expresses endogenous BCAR3 protein. We

find that our anti-BCAR3 antisera but not preimmune sera can detect immunoreactive material in the cytoplasm of 578T cells (data not shown).

d. After identifying antisera that are appropriate for immunohistochemical analysis, use such antisera to determine the presence of AND-34/BCAR3 in either normal or malignant human breast samples. Determine whether AND-34/BCAR3 is expressed in breast stromal cells (Months 15-36).

Progress: We are now in the process of testing our antisera in primary human breast carcinoma samples. Five breast carcinoma samples heve been tested with either preimmune antisera, anti-CYEK (our older anti-AND-34 antisera that cross-reacts with BCAR3) or the new anti-BCAR3 antisera. No specific immunohistochemical staining was seen. We will continue this effort in the coming year.

e. After identifying antisera that are appropriate for Western analysis, assess levels of expression of AND-34/BCAR3 in lysates from normal or malignant human breast samples. Paired immunohistochemical analysis will be used to estimate stromal cell contribution to AND-34 detected by Western analysis.

Progress: **This portion of work has been carried out successfully, although we hope to accrue further data in the next year** with further pathologic material.. We have obtained from the BMC Pathology Department using an IRB-approved protocol, pathologic specimens of a malignant breast tumor and a benign breast lesion (fibroadenoma). These specimens were lysed and Western analysis was carried out using the polyclonal anti-BCAR3 antisera described above. As a control, we included a p130Cas immunoprecipitate from a cell line known to express BCAR3 (HEK-293-T cells). As shown in Figure 2, we were able to detect high level expression of BCAR3 in the breast carcinoma specimen but very little BCAR3 expression in the fibroadenoma specimen.

Key Research Accomplishments:

First, as outlined above, in this second year of this grant's support, we have demonstrated that both the SH2 domain and the GEF-like domain of BCAR3 are required for BCAR3-mediated anti-estrogen resistance in ZR-75 cells (Figure 1).

Second, we have demonstrated expression of BCAR3 in primary human breast carcinoma samples (Figure 2).

Third, although not included in the Specific Aims of this DOD BCRP grant, we believe that our demonstration that BCAR3 activates Rac1, Cdc42, PAK1 and the cyclin D1 promoter, and that the activation of the cyclin D1 promoter in MCF-7 cells is Rac and PAK1-dependent may ultimately prove to

substantially clarify the signaling pathway by which BCAR3 confers antiestrogen resistance in human breast cancer cell lines (see Appendix A).

Reportable Outcomes: See page proofs of Cancer Research article, in press for October 15th 2003.

Conclusions: See attached article.

References: See attached article.

Appendices: Cai D, Iyer A, Near RI, Felekkis KN, Luo Z, Chernoff J, Albanese C, Pestell RG and Lerner A. AND-34/BCAR3, a GDP exchange factor whose over-expression confers antiestrogen resistance, activates Rac1, Pak1 and the cyclin D1 promoter. Cancer Research (in press).

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[CANCER RESEARCH 63, ●●●–●●●, October 15, 2003]

AND-34/BCAR3, a GDP Exchange Factor Whose Overexpression Confers Antiestrogen Resistance, Activates Rac, PAK1, and the Cyclin D1 Promoter[1]

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ABSTRACT

Fn1-3 **AND-34 is a murine protein that binds by a cdc25-like GDP exchange factor domain to the focal adhesion docking protein p130Cas. Overexpression of either of the human homologues of AND-34 and p130Cas, BCAR3 and BCAR1, respectively, has been reported to induce resistance to antiestrogens in breast cancer cell lines. Here we show that overexpression of AND-34 leads to activation of the Rho family GTPases Cdc42 and Rac. Consistent with these findings, BCAR3 overexpression induced alterations in F-actin distribution and augmented both autophosphorylation and kinase activity of the Cdc42/Rac-responsive serine/threonine kinase PAK1. p130Cas-associated BCAR3 protein was detected in the estrogen-independent breast cancer cell line 578-T, but not in estrogen-dependent MCF7 or ZR-75-1 cells. Stable ZR-75-1 transfectants overexpressing BCAR3, but not vector-only transfectants, grew in the presence of the pure antiestrogen ICI 182,780. Stable transfection with RacV12, a constitutively active form of Rac1, also induced antiestrogen resistance in ZR-75-1 cells. Transient transfection of BCAR3 in estrogen-dependent MCF7 cells induced activation of luciferase constructs containing the**
AQ: A **proximal 1745 or 163 bp but not 66 bp of the cyclin D1 promoter. Such cyclin D1 promoter activation was inhibited by dominant negative forms of Rac1 and PAK1. Overexpression of the PAK1 autoinhibitory domain (residues 83–149) but not an inactive PAK1 autoinhibitory domain point mutant (L107F) also blocked BCAR3-mediated cyclin D1 activation. These studies suggest that AND-34/BCAR3 induces antiestrogen resistance in breast cancer cell lines by a Rac1- and PAK1-dependent pathway.**

INTRODUCTION

AQ: B In studies of thymic negative selection, we cloned AND-34, a
Fn4 widely expressed M_r 95,000 protein with an NH_2-terminal SH2[4] domain and a COOH-terminal amino acid sequence with distant homology to the Ras GTPase subfamily GEF or Cdc25 domain [18% identity and 30% homology (1, 2)]. AND-34 constitutively associates with the focal adhesion protein p130Cas, a docking protein that integrates mitogen- and adhesion-related signaling (2). Truncation mutant analysis demonstrated that the COOH-terminal GEF-like domain of AND-34 associates with the COOH terminus of p130Cas. Transient transfection of AND-34 into Cos cells induced activation of
AQ: C Ral and, to a lesser extent, Rap1 and R-Ras (3).

In an open-ended search for genes that regulate antiestrogen resistance, Dorssers and colleagues performed random retroviral integration into 800,000 ZR-75-1 cells, an estrogen-dependent human breast cancer cell line. Among the resulting 80 tamoxifen-resistant clones, 6 resulted from independent retroviral insertion into the promoter for a novel gene BCAR3. The retroviral insertion was shown to result in the AQ: D up-regulation of BCAR3 expression and growth of such ZR-75-1 cells in the presence of either 4-hydroxy-tamoxifen or ICI 182,780 (4). AND-34 is the murine homologue of BCAR3, sharing 86% identity and 93% homology to BCAR3 at the amino acid level. Remarkably, further analysis of the 80 antiestrogen-resistant clones demonstrated that four of the clones had independent retroviral insertion into the promoter of BCAR1, the human homologue of p130Cas (5). Although the GDP exchange activity of BCAR3 has not been examined, nor has its association with human p130Cas, these seminal studies by Dorssers and colleagues suggest that BCAR3 and BCAR1 constitute part of a signaling cascade that bypasses estrogen dependence in human breast cancer cell lines. In this study, we present evidence that AND-34/BCAR3 confers antiestrogen resistance through a Rac-mediated pathway.

MATERIALS AND METHODS

Muristerone-Inducible Cell Line. EcR-293 cells (Invitrogen) were transfected with a pIND-HA-AND-34 construct, and stable clones were selected with 400 μg/ml G418. Inducibility was confirmed in the presence and absence of 1 μM muristerone.

Anti-BCAR3 Antisera. Rabbits were immunized with a 19-amino acid BCAR3 peptide, YEKQLKPFSKLLHEGREST (QCB, Inc., Hopkinton, MA). The specificity of the resulting peptide-immunopurified antisera (anti-YEK) was verified by immunoblotting whole cell lysates of Cos7 cells transfected with vector or HA-tagged BCAR3 with anti-HA or anti-YEK.

F-Actin Staining. Retrovirally transduced NIH 3T3 cells were fixed in 3.7% formaldehyde in PBS for 10 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The AQ: E cells were incubated with 5 units of Alexa594-phalloidin (Molecular Probes) for 20 min at room temperature, washed twice with PBS, examined with a Nikon Diaphot fluorescent microscope, and photographed with a Hamamatsu digital camera.

AND-34/IRES/GFP MSCV Construct. HA-AND-34, subcloned into the Xho and R1 sites of the retroviral vector pMSCV-IRES-GFP (or vector alone) were transiently cotransfected with pCL-Eco packaging plasmid into BOSC AQ: F cells (6). At 72 h, the medium was collected and filtered through a 0.22-μm filter (Costar), and Polybrene (1.25 μg/ml; American Bioanalytical) was added. NIH 3T3 cells were cultured for 16–20 h with the retrovirus-containing media.

Rho and Ras Family GTPase Pull-Down Assays. The technique for pull-down analysis has been described previously (7). Levels of GTP-bound Rac and Cdc42 were determined with a chimeric GST-PAK-RBD protein derived from subcloning amino acids 70–149 of rat PAK1 (NCB accession AQ: G number P35465) into the *Bam*HI and *Sal*I sites of pGEX (Pharmacia). A GST-Rhotekin-RBD construct for determination of GTP-bound Rho was generously provided by Drs. Xiang-Dong Ren and Martin Schwartz (Scripps

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[2] Both authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Medicine, Section of Hematology and Oncology, Boston Medical Center, Evans Biomedical Research Center, Room 427, 650 Albany Street, Boston, MA 02118. Phone: (617) 638-7530; E-mail: alerner@medicine.bu.edu.

[4] The abbreviations used are: SH, Src homology; GEF, GDP exchange factor; HA, hemagglutinin; PID, PAK1 autoinhibitory domain; GFP, green fluorescent protein; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol 3′-kinase; NF-κB, nuclear factor κB.

Research Institute; Ref. 7). For Ral and Rap1 pull-down assays, RalBP1 and RalGDS-RBD constructs were used. Constitutively activated RalA V23, Rap1A V12 and Rac1 V12 constructs were generously provided by Dr. Lawrence Quilliam (Indiana University School of Medicine).

PAK1 Assays. PAK1 kinase assays were carried out as described by Zhao *et al.* (8).

HA-Tagged BCAR3 Transfectants. A human cDNA clone that lacked the first 36 bp of the BCAR3 open reading frame (Incyte Genomics clone 550495) was amplified by PCR with an oligonucleotide that replaced the missing 36 bp and subcloned into a pcDNAI vector containing a 5′ HA epitope. ZR-75-1 cells were transfected with pBKCMV (allowing selection with G418) along with either pcDNAI or pcDNAI HA-BCAR3. For growth assays, HA-BCAR3 or vector-only transfectants were trypsinized, and single cell suspensions were plated in 100 × 20-mm plastic culture flasks at a density of 750,000 cells/flask in 10% FCS and 100 nM 182,780 (Tocris Cookson Ltd.). Culture medium was changed twice per week. On the 13th day, the cells were trypsinized and counted. A comparable strategy was subsequently used to generate and study FLAG-tagged Rac1 V12 or vector-only transfected ZR-75-1 cells. AQ: H

Cyclin D1 Promoter Luciferase Assays. MCF7 cells were seeded into 60-mm dishes at 50–60% confluence in triplicate overnight. Cells were transfected with either HA-BCAR3 or vector (pcDNA1) and the cyclin D1 full-length (−1745 CD1luc) or deletion constructs (−163CD1luc and −66CD1luc). Constructs driving expression of the GST-tagged PID (residues 83–149) as well as the corresponding functionally inactive leucine 107 to phenylalanine PID mutants were made using the pEBG vector. Total transfected DNA was kept constant by addition of the appropriate "empty" vector of the dominant negative expression plasmid being examined. After 48 h, whole cell lysates were harvested, and luciferase activity was determined with a Promega luciferase assay kit (Madison, WI). Relative luciferase units were calculated by normalizing for total protein.

Statistical Analysis. Data are reported as means ± SE. Comparisons between multiple groups were performed using single-factor ANOVA, and secondary comparisons were performed using the Tukey test. Statistical analysis was performed using the SPSS statistical software package.

RESULTS

AND-34/BCAR3 Overexpression Induces Alterations in Cell Morphology and Activates Rac1, Cdc42, and PAK1. To study the functional effects of AND-34 in cells that do not constitutively overexpress this protein, we obtained stable transfectants of the EcR-293 mesenchymal kidney cell line in which muristerone induced overexpression of HA-tagged AND-34. After treatment of one such clone, HA-A34/#9, with muristerone, HA-tagged AND-34 could be detected easily in whole cell lysates with either an anti-HA monoclonal antibody or with polyclonal anti-AND-34 antisera (Fig. 1*A*). Low levels of a protein immunoreactive with anti-HA and polyclonal anti-AND-34/BCAR3 were detected in the noninduced EcR-293 cells (Fig. 1*A*). After the addition of muristerone, 10–20% of HA-A34/#9 cells developed long cellular processes (Fig. 1*B*). Time-lapse cinematography revealed that such processes developed as the cell body moved, leaving a retraction fiber behind (data not shown). The number of HA-A34/#9 cells with cell extensions greater than 1 cell diameter in length increased from 2.9 to 11.0 cells/high-powered field ($P < 0.001$) after muristerone treatment. Because control immunofluorescence studies of permeabilized cells verified that essentially no uninduced cells and all muristerone-treated cells expressed HA-BCAR3 (data not shown), the presence of long cell extensions in BCAR3-overexpressing HEK-293 cells may vary as a function of cell-cell contact or motility. F1 AQ: I

To examine the effects of AND-34 expression in a model system in which cytoskeletal regulation has been well studied, we transduced NIH 3T3 cells with a MSCV retrovirus that drives expression of both GFP and HA-tagged AND-34 (AND-34-RV) or the same retrovirus lacking HA-AND-34 (CT-RV). When F-actin in the GFP-positive transduced cells was examined by fluorescence microscopy using Alexa594-phalloidin, we noted clear stress fibers and modest numbers of lamellipodia in the CT-RV-transduced cells (Fig. 2, *A* and *B*). In contrast, AND-34-RV-transduced NIH 3T3 cells contained both a reduced number and a less organized pattern of parallel stress fibers (Fig. 2, *C* and *D*). AND-34-RV-transduced cells also showed more pronounced membrane "ruffles" or lamellipodia than CT-RV-transduced cells. As a control, filopodia (Fig. 2*E*) and lamellopodia (Fig. 2*F*) were examined in NIH 3T3 cells transiently cotransfected with plasmids driving the expression of GFP and either constitutively activated CDC42 (Cdc42 L61) or Rac (Rac V12), respectively. As shown in Fig. 2, AND-34-RV-transduced NIH 3T3 cells more closely resembled cells containing activated Rac. No significant morphological alterations were observed in NIH 3T3 cells transiently transfected with RalA V23, a constitutively active form of Ral (data not shown). F2

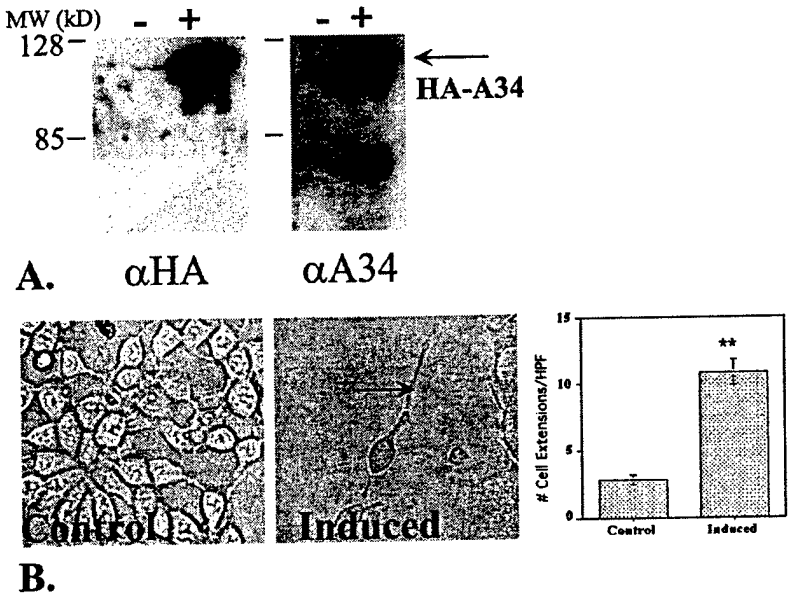

Fig. 1. Induction of AND-34 overexpression in HEK-293 cells is associated with alterations in cell morphology. *A*, an EcR-293 cell line stably transfected with HA-tagged AND-34 (HA-A34/#9) was incubated for 24 h in the presence (+) or absence (−) of 1 μM muristerone, followed by lysis and immunoblot analysis with monoclonal anti-HA antibody (*left panel*) or polyclonal anti-AND-34 antibody (*right panel*). *B*, HA-A34/#9 cells were grown in media with (*Induced*) or without (*Control*) muristerone as per *A*, followed by microscopy. In cultures of similar cell density, the tissue culture plate was divided into eight equal regions, and in each region, the number of cells with cell extensions of >1 cell diameter in length (*right panels*) was quantitated. The observed increase in cell extensions (*column graph* at *bottom right*) was significant at 0.01% (**) by Student's *t* test.

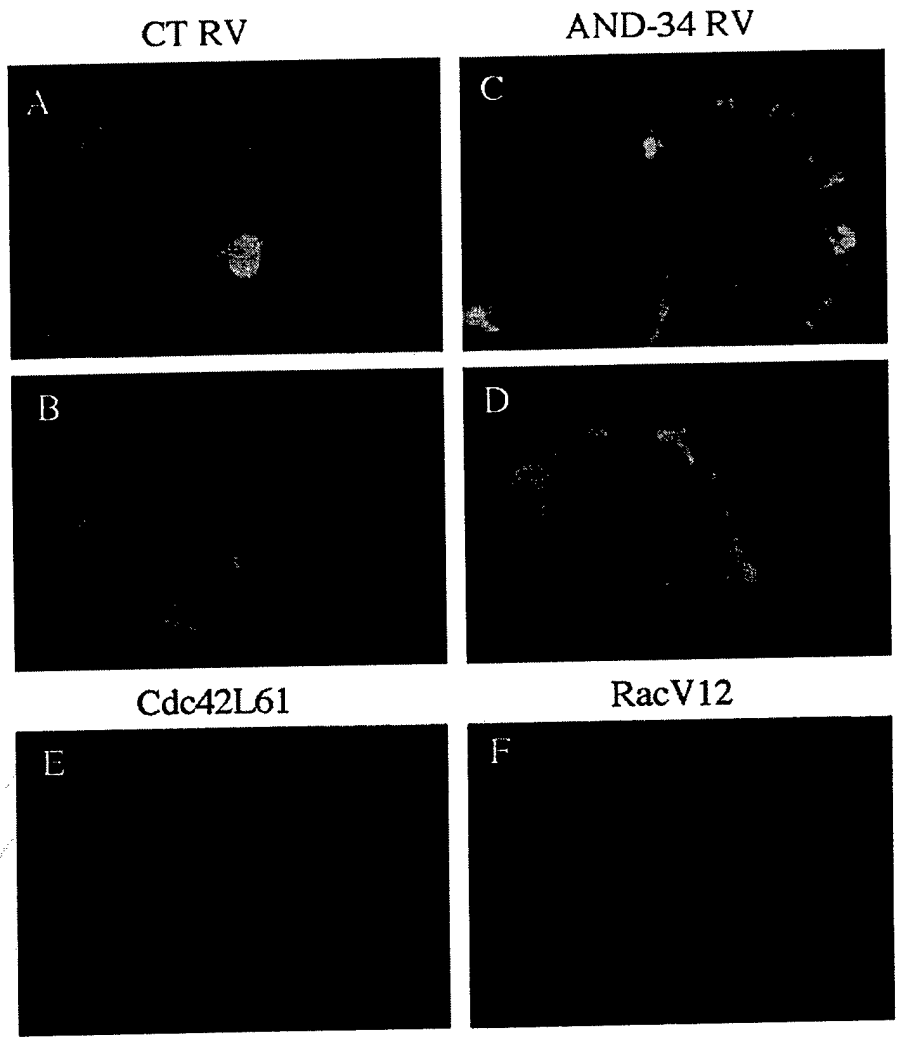

Fig. 2. Overexpression of AND-34 in NIH 3T3 cells results in membrane ruffling and loss of F-actin stress fibers. NIH 3T3 cells were transduced with bicistronic MSCV retroviral constructs expressing GFP without (CT-RV) or with (AND-34-RV) HA-tagged AND-34. GFP-positive cells transduced with CT-RV (*A* and *B*) or AND-34-RV (*C* and *D*) were examined for F-actin distribution by immunofluorescent microscopy of cells that had been fixed and stained with Alexa594-phalloidin. As a control, comparable analysis was carried out on GFP-positive NIH 3T3 cells that had been transiently transfected with constructs driving the expression of GFP and either constitutively active Cdc42 (Cdc42 L61) or Rac (Rac V12; *E* and *F*).

Because similar morphological changes have been observed after overexpression of activated Rho family GTPases, we performed pull-down assays to examine such activation in HA-A34/#9 cells induced to overexpress AND-34 (9, 10). We used a GST-PAK-RBD construct to selectively isolate the GTP-bound forms of transiently transfected FLAG epitope-tagged Cdc42 and Rac1. A GST-Rhotekin-RBD construct was used to isolate endogenous GTP-bound HA-tagged RhoA (7). Significant levels of GTP-bound Cdc42 (Fig. 3*A*) and Rac (Fig. 3*B*) but not RhoA (Fig. 3*C*) were present in induced HA-A34/#9 cells, but not in noninduced HA-A34/#9 cells. In five of six experiments, transient transfection of BCAR3, the human homologue of AND-34 (86% identity), induced activation of endogenous Rac1 in 293T cells (Fig. 3*D*). The Rac1 activation observed in these experiments was less than that in the inducible cell line system, most likely due to a lower percentage of cells expressing BCAR3 after transient transfection as well as relatively high levels of activated endogenous Rac1.

Given our prior findings that AND-34 augmented the ratio of GTP:GDP-bound Ral and Rap1 in Cos7 cells, we sought to determine whether these GTPases might be responsible for the Rac activation we observed in muristerone-treated HA-A34/#9 cells. Transfection of 293T cells with constitutively active forms of these GTPases, RalA V23 and Rap1A V12, failed to induce Rac activation as judged by pull-down assays (Fig. 3*E*). As a control, Ral and Rap1 pull-down assays confirmed augmented levels of GTP-bound RalA and Rap1A in 293T cells transfected with these constitutively active constructs (Fig. 3*E*). These studies suggest that AND-34- and BCAR3-mediated Rac activation occurs independently of Ral or Rap1.

GTP-bound Rac and Cdc42 bind to a CRIB domain in the p21-activated serine threonine kinase PAK1 (11), inducing PAK1 autophosphorylation at serines 199 and 204 and augmenting kinase activity. We therefore examined PAK1 in 293T cells transiently transfected with wild-type BCAR3 and a GST-PAK1 expression construct. After transfection, GST-PAK1 was isolated with glutathione-Sepharose beads and examined for both phosphorylation status and kinase activity. As judged by kinase assay using myelin basic protein as a substrate, overexpression of BCAR3 led in a dose-dependent fashion to GST-PAK kinase activation, as did cotransfection with constitutively active Rac V12 (Fig. 4*A*). Similarly, using a phosphospecific monoclonal antibody, overexpression of BCAR3 augmented levels of GST-PAK1 autophosphorylated at serines 199 and/or 204 (Fig. 4*A*). BCAR3 or Rac V12 transfection particularly augmented levels of a more slowly migrating GST-PAK1 species, consistent with phosphorylation of additional residues. Western analysis of whole cell lysates demonstrated comparable expression levels of GST-PAK1 in the presence or absence of cotransfected BCAR3. In contrast, transfection

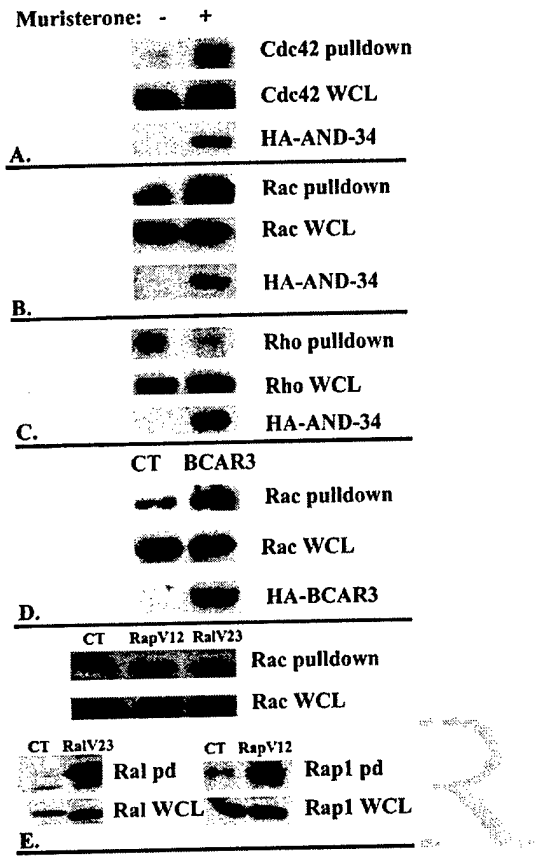

Fig. 3. Overexpression of HA-AND-34 and BCAR3 induces activation of Rac and Cdc42 in EcR-293 cells. *A–C*, HA-AND-34/#9 cells were transfected with constructs driving the expression of FLAG epitope-tagged Cdc42 (*A*), FLAG-tagged Rac (*B*), or HA-tagged Rho (*C*); allowed to recover for 24 h; and then incubated in media with or without 1 μM muristerone for an additional 24 h. To measure the level of GTP-bound GTPases, whole cell lysates were incubated with either GST-PAK (Cdc42 and Rac) or GST-Rhotekin RBD (Rho). After isolation of the GST-chimeric proteins with glutathione beads, eluted proteins were immunoblotted with anti-FLAG (Cdc42 and Rac) or anti-HA (Rho). To confirm equal expression of the epitope-tagged GTPases with or without muristerone treatment, aliquots from the same whole cell lysates (*WCL*) were also immunoblotted with these antibodies as shown. To confirm induction of HA-tagged AND-34, similar aliquots were immunoblotted with anti-HA antibody as shown. *D*, 293T cells were transiently transfected with constructs driving the expression of BCAR3 or vector alone, followed by pull-down analysis for GTP-bound endogenous Rac as described above. *E*, 293T cells were transiently transfected with constructs driving the expression of constitutively active Rap (*RapV12*) or Ral (*RalV23*), followed by pull-down analysis for activated Rac, Ral or Rap, as indicated.

with constitutively active Rac V12 diminished levels of GST-PAK1 (Fig. 4*A*).

BCAR3 Associates with Human p130Cas in an Estrogen-Independent Breast Cancer Cell Line. To examine the potential role of Rac or Cdc42 activation in BCAR3-mediated antiestrogen resistance, we examined two human breast cancer cell lines, 578-T and ZR-75-1. As reported previously, 578-T cells were resistant to the effects of antiestrogens, as judged by the absence of any alteration in the fraction of cells in S-G_2-M phase after culture for 72 h in the presence or absence of 100 nM ICI 182,780, a pure estrogen receptor antagonist (data not shown). In contrast, comparable treatment of ZR-75-1 cells resulted in a 73% reduction in the fraction of cells in S-G_2-M. Northern analysis demonstrated high-level expression of BCAR3 transcripts in 578-T cells, whereas in ZR-75-1 cells and MCF7 cells, another estrogen-dependent human breast cancer cell line, low levels of BCAR3 transcripts were detectable only after prolonged autoradiograph exposure (data not shown). To determine whether the augmentation in BCAR3 transcript levels was associated with comparable changes in BCAR3 protein expression, we developed a polyclonal antisera raised against a BCAR3 peptide. An anti-BCAR3 immunoreactive M_r 100,000 protein was detected in p130Cas (BCAR1) immunoprecipitates from 578-T cells, but not from ZR-75-1 cells (Fig. 4*B*). This result suggests that, as is the case for AND-34 and p130Cas in murine cells, at least a fraction of BCAR3 is constitutively associated with p130Cas (BCAR1) in human 578-T cells.

To study the effect of BCAR3 on antiestrogen sensitivity in ZR-75-1 cells, we generated a plasmid construct in which an NH_2-terminal HA epitope-tagged full-length BCAR3 cDNA was driven by a constitutive cytomegalovirus promoter. Stable transfectants of ZR-75-1 cells were obtained with both HA-tagged BCAR3 (BCAR3 #2, #4, and #6) and with the vector alone (CT #1, #3, and #4). The three BCAR3-overexpressing ZR-75-1 stable transfectants contained significantly greater numbers of long, branched cellular processes than the three vector-only transfectants ($P < 0.0001$ by one-factor ANOVA). As expected, none of the three vector-only stable transfectants grew in 100 nM ICI 182,780. In contrast, all three of the BCAR3-overexpressing clones grew well in 100 nM ICI 182,780 (Fig. 4*C*). The cell doubling time of the three BCAR3-overexpressing transfectants was not significantly different from that of the three vector-only transfectants when grown in normal media (data not shown). After the addition of antiestrogen to the culture media, growth of all of the clones gradually diminished over the first week. Subsequently, however, the growth rate of the BCAR3-overexpressing clones increased such that they were growing at close to (BCAR3 #2 and #6) or moderately slower than (BCAR3 #4) the doubling time of cells grown in the absence of the antiestrogen (data not shown). To establish whether the ability of BCAR3 to activate Rac1 might underlie its ability to confer antiestrogen resistance to ZR-75-1 cells, we isolated stable transfectants of this cell line containing either a constitutively active form of Rac1, FLAG-tagged Rac1 V12, or vector alone. Western analysis confirmed overexpression of Rac V12. Whereas the three control vector lines failed to grow in 100 nM ICI 182,780, the two Rac1 V12 cell lines grew robustly (Fig. 4*D*).

BCAR3 Overexpression Activates the Cyclin D1 Promoter in a Rac- and PAK1-dependent Manner. Cyclin D1 is known to play an important role in estrogen-mediated breast cancer cell growth, and antiestrogens block cyclin D1 expression. To analyze the effects of BCAR3 on cyclin D1 expression in transient transfection assays, we used a second estrogen-dependent cell line, MCF7, that allowed greater transfection efficiency than ZR-75-1 cells. By Western analysis, transient transfection of BCAR3 into MCF7 cells augmented cyclin D1 expression (Fig. 5*A*). To determine whether BCAR3 could F5
activate the cyclin D1 promoter, we used a construct in which a 1745-bp genomic fragment containing the cyclin D1 promoter drives luciferase expression. In five experiments, cotransfection of the cyclin D1 construct with BCAR3 markedly augmented luciferase activity relative to cotransfection with empty vector, regardless of the presence or absence of 100 nM ICI 182,780 (Fig. 5*B*). To determine the AQ: K
minimal promoter length required for BCAR3-mediated cyclin D1 transcriptional activation, we examined two additional cyclin D1 luciferase constructs containing 163 or 66 bp 5′ of the cyclin D1 transcriptional initiation site. Whereas BCAR3 augmented transcription of the 1745- and 163-bp constructs, no significant transcriptional activation of the 66-bp construct was detected (Fig. 5*C*). Thus, transcription factor binding sites 5′ of bp 66 are likely to play a critical role in BCAR3-mediated cyclin D1 promoter activation.

Because the work described above suggests that overexpression of AND-34 or BCAR3 activates Rac and the serine/threonine kinase PAK1, we sought to determine whether such activation might play a role in BCAR3-mediated cyclin D1 promoter activa-

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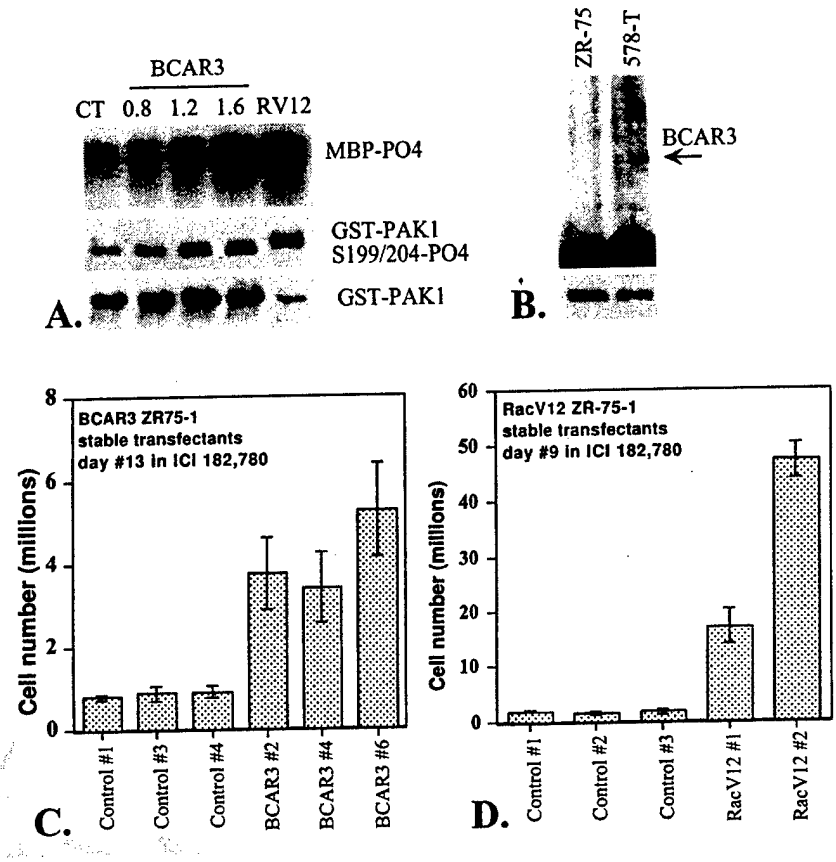


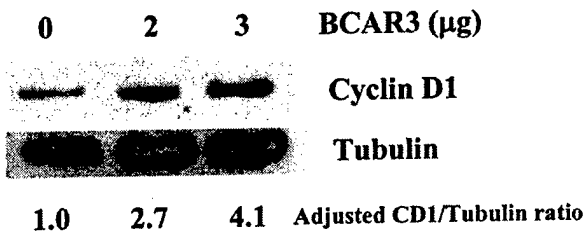
Fig. 4. Overexpression of BCAR3 activates the serine/threonine kinase PAK1, and ZR-75-1 cells stably transfected with constitutively active Rac, like BCAR3, are able to grow in the presence of the antiestrogen ICI 182,780. *A, top panel,* 293T cells were transiently transfected with constructs driving the expression of GST-PAK1 alone (*CT*) or in combination with varying quantities (shown in μg) of BCAR3 or a constitutively active Rac V12 (*RV12*). GST-PAK was isolated with glutathione-Sepharose beads and subjected to an *in vitro* kinase assay using myelin basic protein (*MBP*) as a substrate. An autoradiograph of the kinase reaction separated by PAGE is shown. *A, middle panel,* an aliquot of the purified GST-PAK from the same transfections was immunoblotted with a monoclonal antibody specific for PAK1 autophosphorylated at serine 199 or 204. *A, bottom panel,* comparable expression of GST-PAK in the transfections was assessed by immunoblotting for PAK1. *B,* ZR-75-1 and 578-T cells were analyzed for the presence of p130Cas-associated BCAR3 protein by p130Cas immunoprecipitation and BCAR3 immunoblot analysis. *C,* cumulative cell numbers of three vector-only and three HA-AND-34 ZR-75-1 transfectants 13 days after addition of 100 nM ICI 182,780 to tissue culture media. *D,* cumulative cell numbers of three vector-only and two FLAG-tagged RacV12 ZR-75-1 transfectants 9 days after addition of 100 nM ICI 182,780 to tissue culture media.

tion. We cotransfected BCAR3 and the 1745-bp cyclin D1 luciferase construct with either control vector, dominant negative Rac1(N17), or dominant negative PAK1(K297A) (12). Both Rac1(N17) ($P < 0.008$) and PAK1(K297A) ($P < 0.001$) reduced BCAR3-mediated cyclin D1 promoter activation (Fig. 5, *D* and *E*, respectively). In contrast, cotransfection with a dominant negative form of MEK1(S218A,S222A) had no inhibitory effect on BCAR3-mediated cyclin D1 promoter activation (Fig. 5*F*; Ref. 13). Given that the dominant negative PAK construct used has an intact CRIB domain that could theoretically sequester activated Cdc42 and Rac from other potential effector proteins, we next cotransfected BCAR3 with PID (residues 83–149) that does not bind Rac or Cdc42 but can inhibit PAK1 activation (8). BCAR3-mediated cyclin D1 promoter activation was markedly inhibited by the wild-type PID construct, but not by a PID construct inactivated by replacing leucine 107 with phenylalanine (Fig. 5*G*; Ref. 14). These experiments suggest that BCAR3-mediated antiestrogen resistance may be mediated, at least in part, by Rac and PAK1-induced cyclin D1 promoter activation.

DISCUSSION

In this study, we demonstrate that overexpression of AND-34 and BCAR3 can activate Rac and/or Cdc42 signaling pathways. Given that AND-34 and BCAR3 have domains homologous to the cdc25 AQ: L domain of GEFs, it is likely that such Rho family GTPase activation occurs indirectly as a result of initial activation of a Ras subfamily GTPase. However, although there is ample precedent for cross-talk between Ras and Rho subfamily GTPases, to our knowledge this is the first report in which overexpression of a GEF with a cdc25-like domain (but no Dbl domain) results, directly or indirectly, in activation of Rho family GTPases (15, 16). One potential mechanism by which a Ras subfamily GEF might indirectly activate Rho subfamily GTPases is through activation of PI3K. The Rac/Cdc42 GEF activity of several Dbl domain-containing GEFs such as Sos-1, Vav, and PIX is activated by interaction of their PH domains with PIP3, the product AQ: M of PI3K (17–20). Alternatively, AND-34 or BCAR3 overexpression could lead to kinase activation and the phosphorylation and activation of a Rho family GEF. Tyrosine phosphorylation of the Dbl family GEF Vav occurs after stimulation of some 35 membrane receptors and has been reported to induce either activation or down-modulation of Vav activity (21, 22). We are currently examining whether AND-34 or BCAR3 overexpression activates PI3K or leads to tyrosine phosphorylation of known Rac/Cdc42 GEFs.

AND-34/BCAR3 could induce Rho subfamily GTPase activation by acting as an adapter protein rather than as a GEF. AND-34 and BCAR3 contain both an SH2 domain and a proline-rich region that could serve to recruit either a Rho subfamily GEF or an enzyme that activates such a GEF. As discussed above, overexpression of p130Cas as well as BCAR3 results in tamoxifen resistance, raising the question of whether p130Cas achieves this effect by regulating the GEF activity of BCAR3 or through another protein. Several reports have linked p130Cas to Rac1 activation (reviewed in Ref. 23). p130Cas overexpression enhances epithelial cell migration through a CrkII- and Rac1-dependent pathway (24). Whereas the SH2 domain of CrkII binds inducibly to tyrosine-phosphorylated p130Cas, the SH3 domain of CrkII binds to several proteins including the Rap1 GEF C3G and DOCK180 (25, 26). DOCK180 binds Rac1, but not RhoA or Cdc42, and DOCK 180 overexpression leads to increased levels of GTP-



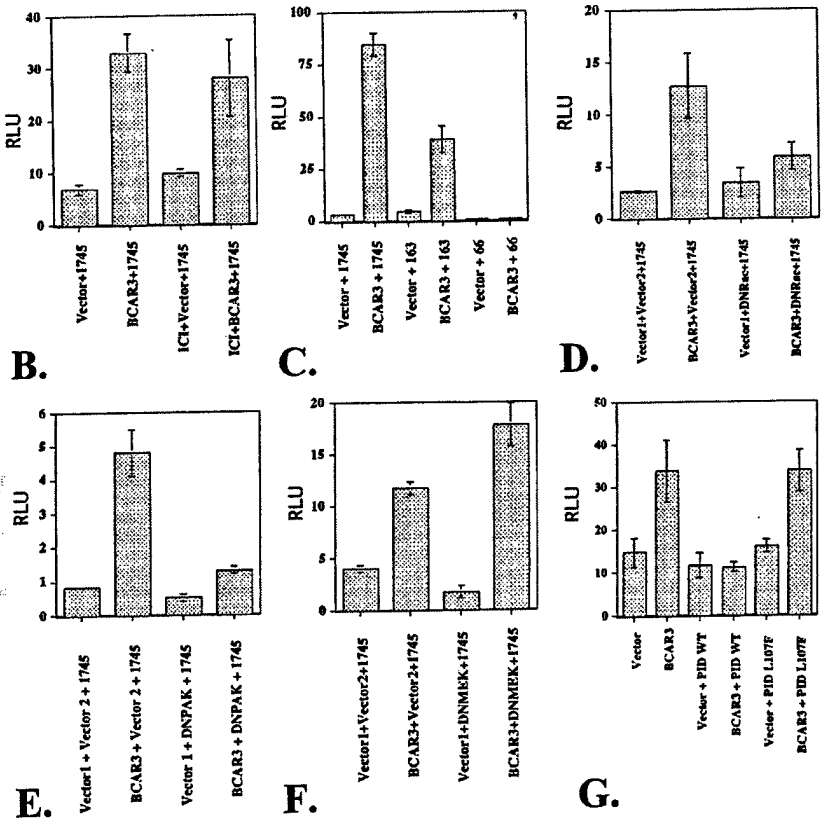


Fig. 5. Overexpression of BCAR3 in MCF7 cells augments cyclin D1 levels and activates transcription from the cyclin D1 promoter. *A*, MCF7 cells were lysed 48 h after transient transfection with a construct driving expression of BCAR3, followed by immunoblot analysis for cyclin D1. The cyclin D1 band density relative to the control sample is shown below the immunoblot, after adjustment for the density of the corresponding tubulin band. *B*, MCF7 cells, cultured in either the presence or absence of 100 nM ICI 182,780 for 24 h, were transiently transfected with luciferase constructs driven by a 1745-bp fragment of the cyclin D1 promoter, as indicated, along with BCAR3 or its vector. Twenty-four h after transfection, whole cell lysates were assessed for luciferase expression by luminometer. Whole cell lysates were assayed for protein content, and the luciferase data were correspondingly normalized. *C*, MCF7 cells were transiently transfected with luciferase constructs driven by 1745-, 163-, or 66-bp fragments of the cyclin D1 promoter, as indicated, along with BCAR3 or its vector. *D–F*, MCF7 cells were transfected with a luciferase construct driven by a 1745-bp fragment of the cyclin D1 promoter, with or without BCAR3, and with or without constructs of dominant negative Rac1(N17), dominant negative PAK1(K297A), dominant negative MEK1(S218A,S22A), or equivalent amounts of their corresponding empty vectors, as indicated. *G*, as for *D–F*, except that MCF7 cells were transfected with or without the autoinhibitory domain of PAK1 (*PID WT*) or a PID construct inactivated by mutation of leucine 107 to phenylalanine (*PID L107F*).

bound Rac (27). Given this background, it is possible that p130Cas overexpression could induce Rac activation by pathways independent of BCAR3. However, our observation of Cdc42 activation by AND-34 overexpression suggests that AND-34/BCAR3-mediated Rho subfamily GEF activation is unlikely to be occurring by a DOCK180-mediated pathway. Finally, AND-34/BCAR3 might act directly as a Rac/Cdc42 GEF. Although AND-34/BCAR3 has homology to cdc25, the homology is distant enough (18% identity) to raise the possibility that this enzyme may have dual Ras and Rho family GEF activity. Studies examining the *in vitro* GEF activity of AND-34 will be required to resolve this question unequivocally.

A number of reports have previously implicated Rac in regulation of the cyclin D1 promoter, supporting our hypothesis that Rac itself is responsible for BCAR3-mediated antiestrogen resistance. Studies using bovine tracheal myocytes, NIH 3T3 cells, HeLa cells, and Cos1 cells have reported that Rac activation can induce cyclin D1 synthesis and progression through G_1 by a superoxide- and NF-κB-mediated pathway (28–31). Although these studies have implicated p67phox as a *necessary* effector for Rac-mediated cyclin D1 promoter activation, they have not shown that activation of this p67/superoxide/NF-κB pathway is *sufficient* for this end point. In fact, the multiple transcription factor binding elements that have been shown to be required for cyclin D1 promoter activation would suggest that robust activation of this promoter requires the synchronous activation of several signal transduction pathways. Our examination of BCAR-mediated effects on a series of cyclin D1 promoter deletion constructs suggests that critical transcription factor-binding elements are contained in the region from −66 to −163. Prior studies have documented several functionally important DNA transcription factor-binding sites in this region, including TCF/LEF (−75), E2F (−127), and SP1 [−143 (32–34)]. We are currently examining the role of these transcription factors, as well as the p67/superoxide/NF-κB pathway, in BCAR3-mediated cyclin D1 promoter activation.

Van der Flier *et al.* (35) have examined the expression of BCAR1 (p130Cas) in breast cancer specimens and found that overexpression of BCAR1 was associated with short disease-free interval and survival. Examination of BCAR3 in normal and malignant human breast specimens will be necessary to determine whether BCAR3 overexpression induces antiestrogen resistance in patients with breast cancer. Regardless of the outcome of such studies, our work suggests that activation of a Rac and PAK1 signaling pathway may play a role in antiestrogen resistance in human breast cancer.

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